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Kinetics of Thiamin Cleavage by Sulphite

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Results are presented on the rate of thiamin cleavage by sulphite in aqueous solutions as affected by temperature (20–70°), pH (2·5–7·0), and variation of the concentration of either thiamin (1–20 μM) or sulphite (10–5000 μM as sulphur dioxide). Plots of the logarithm of percentage of residual thiamin against time were found to be linear and cleavage thus was first-order with respect to thiamin. At pH 5 the rate was also found to be proportional to the sulphite concentration. In the pH region 2·5–7·0 at 25° the rate constant was 50 M^{-1} $hr.^{-1}$ at pH 5·5–6·0, and decreased at higher or lower pH values. The rate of reaction increased between 20° and 70°, indicating a heat of activation of 13·6 kcal./mole.

Williams (1935), Williams, Waterman, Keresztesy & Buchman (1935*a*) and Williams, Buchman & Ruehle (1935*b*) introduced the method of cleavage of thiamin by bisulphite to establish its structure. Although they identified the products of the reaction they did not investigate the mechanism or the factors affecting the rate and extent of cleavage. Williams *et al.* (1935*a*), however, did report that pH markedly influenced the rate and extent of thiamin cleavage. At pH 5·0 the cleavage was complete in 24–48 hr. at room temperature, whereas with aqueous sulphurous acid the cleavage was much slower, a 50% yield being obtained only after 6 months. Lhoest, Baumann & Busse (1957) confirmed the stoicheiometric cleavage at pH 4·8 and also the identity of the cleavage products. Matsukawa & Yurugi (1952*a*,*b*) and Kawasaki, Suhara & Horio (1958*a*,*b*) also investigated this cleavage. We observed (Joslyn & Leichter, 1968) that residual sulphite present in some commercially available preparations of vitamin-free casein was largely responsible for the cleavage of thiamin during storage in aqueous suspensions, both at 25° and when frozen at −15°. In the present paper we report a systematic study of the rate of thiamin cleavage by sulphite in aqueous solutions as affected by temperature, pH and the relative concentration of either thiamin or sulphite.

MATERIALS AND METHODS

Thiamin hydrochloride was a U.S.P. preparation obtained from Calbiochem, Los Angeles, Calif., U.S.A., and the sodium metabisulphite was a reagent-grade chemical from Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.

Thiamin was determined by the thiochrome method of the Association of Vitamin Chemists (1966) with the enzyme-digestion and column-purification steps omitted. Suitable dilutions of the test solution were made to permit the reading of the fluorescence on the Coleman model 12A photofluorimeter. Sulphur dioxide determinations were made by the colorimetric method described by Nury, Taylor & Brekke (1959). The concentrations of thiamin in unbuffered solutions were either 6 or 10 μM, and the concentrations of sulphite were either 2000 or 6200 μM. The temperature was maintained at 25±0·5° by a thermostatically controlled water bath, and the pH was adjusted to 5·0±0·05 with dilute NaOH or HCl, except where these factors were used as variables. The conditions for each reaction are shown in the respective figures and tables. At the conclusion of each experiment the pH was again determined in the solution used and in almost every case the change in pH was within the limits of experimental error. To prevent oxidation of the sulphite in the solution, freshly boiled water cooled under a stream of N_2 was used as solvent. This was used to prepare stock solutions of sulphite or thiamin. These solutions were pipetted into glass-stoppered 250 ml. Erlenmeyer flasks previously flushed out with N_2, but the gas was not passed through the reaction mixture during the test. Residual sulphite concentrations were measured at the end of each experiment. No measurable losses of sulphite were found except when its initial concentration was very low, e.g. at 10 μM-SO_2 a loss of 15% occurred after 12 hr. The stability of thiamin in an aqueous solution in the absence of sulphite was also determined under the various conditions of the experiment and it was found to remain stable.

To determine possible interference of sulphite in the thiochrome method three concentrations of sulphite (200, 600 and 1500 μg./100 ml., as SO_2) were added to the acid 25% (w/v) KCl solution containing thiamin (200 μg./100 ml.). There was no interference with the assay and the recovery of added thiamin was not affected by sulphite at the concentrations used.

Each of the values in the graphs and tables represents the average of closely agreeing duplicate analyses. Variations between duplicate samples rarely exceeded 3%.

The specific pseudo-unimolecular reaction-rate constants were calculated from the equation:

$$k_1 = \frac{2{\cdot}303}{t} \log \frac{a}{a-x}$$

where t is time (hr.), a is the initial concentration of thiamin, x is the amount of thiamin cleaved in time t, and $(a-x)$ is the concentration remaining after time t. The overall bimolecular constant was expressed as k_1c, where c is the molar concentration of sulphite.

The cleavage products of thiamin by sulphite were identified by t.l.c. (Waring, Goad & Ziporin, 1968).

RESULTS

Effect of thiamin concentration on cleavage of thiamin by sulphite. Various initial concentrations of thiamin (1–20 μM) in aqueous solutions at pH 5·0 and 25° were treated with 2000 μM-sulphite. When logarithms of the percentages of residual thiamin were plotted against time, the resulting curves were linear for 11 hr. (Fig. 1). The calculated specific reaction-rate constants for the various thiamin concentrations shown in Table 1 are essentially identical.

In a typical solution containing 10 μM-thiamin and 1000 μM-sulphur dioxide at pH 5·0 and 25°, when the percentage of residual thiamin is plotted for zero-, first- and second-order reaction (Fig. 2) the resulting graph for the first-order reaction is linear over a 12·5 hr. period. Deviations from linearity, however, occur for the zero- and second-order reaction plots. These results indicate that in the cleavage of thiamin by sulphite, in an aqueous solution at pH 5·0 and 25° and at the concentrations of the reactants tested, the overall reaction is unimolecular with respect to thiamin. Farrer (1945) and Farrer & Morrison (1949) also found that the destruction of thiamin in boiling solutions was a first-order reaction with respect to thiamin. The specific reaction-rate constant they reported, however, varied with pH and type of buffer.

Sulphite cleavage of thiamin as a function of sulphite concentration. The cleavage of thiamin at an initial concentration of 10 μM and at pH 5·0 and 25° by sulphite is first-order at sulphur dioxide concentrations in the range 10–5000 μM. The rate constant of thiamin cleavage increases linearly about 40-fold with the increase of sulphite concentration from 100 to 5000 μM. As shown in Fig. 3, the plot of k_1 against sulphite concentration is a straight line through the origin. From this graph the bimolecular rate constant for the reaction is 45 $M^{-1}hr.^{-1}$ at pH 5·0 and 25°.

Effect of pH *on sulphite cleavage of thiamin.* In the pH range 2·5–7·0 at 25° the rate of cleavage of thiamin increases with pH up to pH 5·5–6·0 and

Table 1. *Specific reaction-rate constants for cleavage of thiamin by sulphite at* 2000 μM *(as sulphur dioxide) in aqueous solutions at* pH 5·0 *at* 25° *as a function of thiamin concentration*

Concn. of thiamin (μM)	Bimolecular rate constant ($M^{-1}hr.^{-1}$)
1	47
2	47·5
5	46·5
10	47·5
20	47

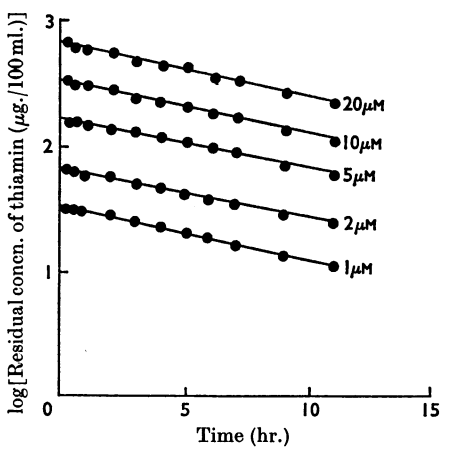


Fig. 1. Rate of cleavage of thiamin at the various concentrations indicated on the figure by sulphite (2000 μM as SO_2) at pH 5·0 at 25°.

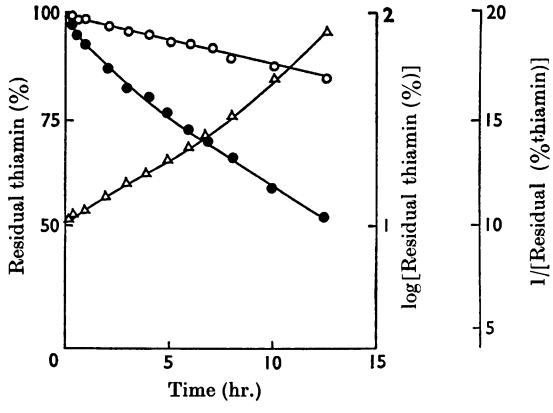


Fig. 2. Cleavage of thiamin (10 μM) by sulphite at 1000 μM (as SO_2) at pH 5·0 at 25° plotted as: ●, zero-order reaction, residual thiamin (%); ○, first-order reaction, log[residual thiamin (%)]; △, second-order reaction, 1/[residual thiamin (%)].

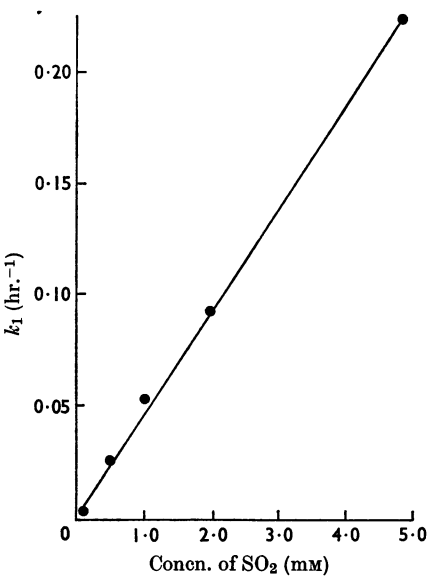


Fig. 3. Pseudo-unimolecular rate constant at pH 5·0 and 25° as a function of sulphur dioxide concentration.

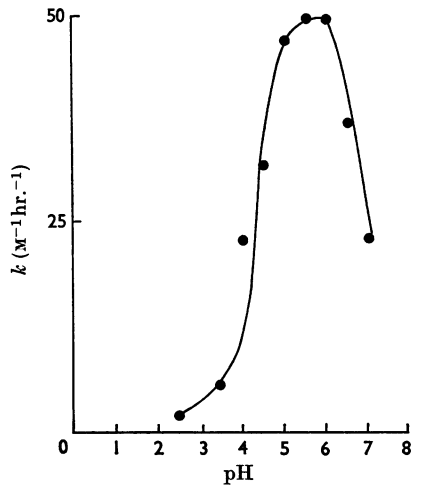


Fig. 4. Bimolecular reaction-rate constants as a function of pH. Experimental details are given in the text.

then decreases. Plots of logarithm of percentage of residual thiamin against time are linear over the pH range 2·5–7·0 and the cleavage thus is first-order with respect to thiamin in this pH range. From the plot of bimolecular rate constants against pH (Fig. 4) the rate constant at pH 5·5 is about 30 times that at pH 2·5.

Thiamin cleavage by sulphite as a function of temperature. Plots of the logarithm of percentage residual thiamin against time are linear at each temperature over the range 20–70°. The rate of thiamin cleavage is doubled for a 10° rise in this range (Table 2). Fig. 5 shows that log$k$ varies linearly with $1/T$. The specific rate constant of thiamin cleavage by sulphite in unbuffered solution thus follows the typical Arrhenius equation. The activation energy for the reaction is 13·6 kcal./mole. The approximate value calculated from the data of Farrer & Morrison (1949) presented graphically for the thermal destruction of thiamin was of the same order of magnitude (24·5 kcal./mole). Goldblith, Tannenbaum & Wang (1968) reported that the Arrhenius constant for destruction of thiamin by heat and by microwave energy was similar, and the constant calculated from the data they presented was approx. 20·0 kcal./mole.

Table 2. *Specific reaction-rate constants of cleavage of 10 μM-thiamin by sulphite at 2000 μM (as sulphur dioxide) at pH 5·0 at 25° as a function of temperature*

Temp.	Bimolecular rate constant (M^{-1}hr.$^{-1}$)
20°	35
30	65
40	145
50	310
60	520
70	990

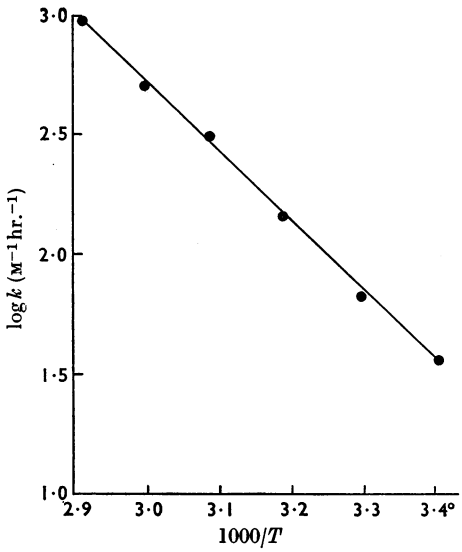
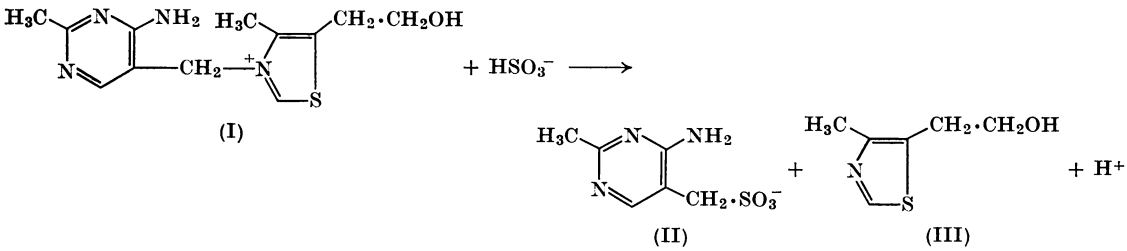


Fig. 5. Arrhenius plot of log (rate constant for thiamin cleavage by sulphite) versus reciprocal of absolute temperatures.



(I) (II) (III)

Scheme 1. (I) Thiamin; (II) (6-amino-2-methylpyrimid-5-yl)methanesulphonic acid; (III) 5-β-hydroxyethyl-4-methylthiazole.

DISCUSSION

The demonstration that the cleavage of thiamin by sulphite is first-order in each reactant is consistent with a typical nucleophilic displacement as shown in Scheme 1. Kawasaki *et al.* (1958*a*) reported that the reaction occurred with thiamin (I) but not with thiothiamin.

Since the nitrogen atom in the thiazole ring of thiamin is quaternary, but is tertiary in the thiazole ring of thiothiamin and in the thiazole ring of the thiol form of thiamin in strongly alkaline solution (in which the latter is not cleaved by sulphite), Kawasaki *et al.* (1958*a*) concluded that a quaternary nitrogen atom is required for this cleavage.

Bonvicino & Hennessy (1957) showed, however, confirmed by Torrence & Tieckelmann (1968), that cleavage of thiamin still occurred after reduction of the thiazole ring by borohydride. This reduction would be expected to convert the nitrogen atom into a worse leaving group. The peculiar catalytic effect of aniline (Matsukawa & Yurugi, 1952*a*,*b*) may bear some relation to its catalysis of nucleophilic addition to carbonyl compounds (Cordes & Jencks, 1962).

Kawasaki *et al.* (1958*a*,*b*) presented results on the cleavage of thiamin by sulphite in the absence and presence of oxygen. Their results when plotted also indicate that the reaction was first-order but only for the first 24hr. They showed that thiamin was cleaved by sulphite more rapidly in a solution saturated with nitrogen than in one saturated with oxygen, but they gave no evidence on whether the dissolved oxygen stabilized the thiamin or decreased the concentration of sulphite by oxidation.

In the overall reaction, 1 mole of thiamin (I) reacts with 1 mole of sodium sulphite to yield the sparingly soluble pyrimidine sulphonic acid (II) and a chloroform-soluble basic product, 5-β-hydroxyethyl-4-methylthiazole (III). This is a nucleophilic displacement reaction in which the sulphur atom of sulphite is oxidized to the sulphate of the sulphonic acid group and the nitrogen atom of the thiazole moiety is reduced from the quinquevalent to the tervalent state. Cecil (1963) gives an analogous nucleophilic displacement reaction of sulphite with disulphide:

$$R_1 \cdot S \cdot S \cdot R_2 + SO_3^{2-} \rightleftharpoons R_1 \cdot S \cdot SO_3^- + R_2 \cdot S^-$$

The pH-dependence of the cleavage of thiamin may be due as much to the different reactivities of the molecular and ionic species present in aqueous sulphurous acid as to the possible effects of pH on thiamin itself. The results reported by Maier & Metzler (1957) indicate that the pseudo-base form of thiamin is not formed in appreciable concentration below pH 11. The data published on the dissociation of sulphurous acid are variable, but *Gmelins Handbuch* (1960) gives constants that correspond to pK values of 1·8 and 7·0. The former is too low to explain the fall in rate on the acid side; this fall may be connected with the pK 4·5 of thiamin (see Williams & Ruehle, 1935). Almost all the sulphurous acid is present as HSO_3^- at the optimum pH of the reaction. The fact that the rate of cleavage slows as the pH rises over the range where HSO_3^- is converted into SO_3^{2-} suggests that SO_3^{2-} may contribute little to the cleavage. This would be surprising, since SO_3^{2-} is more nucleophilic than HSO_3^- in the reaction with formaldehyde or with disulphides. Possibly there is some feature in the thiamin molecule that prevents the bivalent SO_3^{2-} from reacting with it. But it is also possible that SO_3^{2-} does play a part in the reaction even though it is a minor form at the optimum pH, and that the pH-dependence is determined by the participation of protons in some other way.

Kawasaki *et al.* (1958*a*) reported that sulphite cleavage of thiamin is very slight in dilute solutions whereas conversion of thiothiamin into thiamin takes place only in a dilute solution of thiothiamin. Under our conditions appreciable cleavage of thiamin required a considerable excess of sulphite, as well as a pH about 5. At extremely low concentrations of sulphite (10μM as sulphur dioxide) and at low pH values (2·5) the cleavage of thiamin (10μM) by sulphite is negligible.

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